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Research article

Understanding abiotic stress tolerance mechanisms in soybean: A comparative evaluation of soybean response to drought and flooding stress

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ABSTRACT

Many sources of drought and flooding tolerance have been identified in soybean, however underlying molecular and physiological mechanisms are poorly understood. Therefore, it is important to illuminate different plant responses to these abiotic stresses and understand the mechanisms that confer tolerance. Towards this goal we used four contrasting soybean (*Glycine max*) genotypes (PI 567690 – drought tolerant, Pana – drought susceptible, PI 408105A – flooding tolerant, S99-2281 – flooding susceptible) grown under greenhouse conditions and compared genotypic responses to drought and flooding at the physiological, biochemical, and cellular level. We also quantified these variations and tried to infer their role in drought and flooding tolerance in soybean. Our results revealed that different mechanisms contribute to reduction in net photosynthesis under drought and flooding stress. Under drought stress, ABA and stomatal conductance are responsible for reduced photosynthetic rate; while under flooding stress, accumulation of starch granules played a major role. Drought tolerant genotypes PI 567690 and PI 408105A had higher plastoglobule numbers than the susceptible Pana and S99-2281. Drought stress increased the number and size of plastoglobules in most of the genotypes pointing to a possible role in stress tolerance. Interestingly, there were seven fibrillin proteins localized within the plastoglobules that were up-regulated in the drought and flooding tolerant genotypes PI 567690 and PI 408105A, respectively, but down-regulated in the drought susceptible genotype Pana. These results suggest a potential role of Fibrillin proteins, FBN1a, 1b and 7a in soybean response to drought and flooding stress.

1. Introduction

Drought, flooding, high temperature, cold, salinity, and nutrient availability are abiotic factors that have a huge impact on world agriculture and account for more than 50% reduction in average potential yields for most major crops (Wang et al., 2003). As climate prediction models show increased occurrences of drought, flooding, and high temperature spells during the crop growing periods (IPCC, 2008; Mittler and Blumwald, 2010), global food production will continue to be challenged. The demand for food and oil crops will continue to rise with the increase in global population; therefore improving productivity to ensure sustainable yields under changing environmental conditions is essential. To achieve global food security there is a need to increase our understanding of plant responses to abiotic stress with an aim of breeding crops that can maintain higher photosynthetic rates, better growth, and improved yield under stress conditions (Condon et al., 2004; Morison et al., 2008). Some level of success has been achieved in crop breeding for tolerance to abiotic stresses through genetic manipulation of transcription factors (TFs), late embryogenesis abundant (LEA) proteins, and antioxidant proteins (Umezawa et al., 2006; Bhatnagar-Mathur et al., 2008). However, research programs aimed at developing tolerance to a particular stress do not necessarily test susceptibility to other abiotic stresses and this can have unforeseen consequences.

Although irrigation can be used as a strategy to overcome the effects of drought stress on crop yields, the available water

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resources continue to decline. Therefore, adapting crops to water-limited environments and improving their water use efficiency will be crucial for developing climate-resilient cultivars that are capable of producing more food per unit of water used. Drought stress causes tissue dehydration which is characterized by fundamental changes in water relations, physiological and biochemical processes, membrane structure, as well as ultrastructure of subcellular organelles (Sarafis, 1998; Yordanov et al., 2003). At the whole-plant level, drought stress leads to a progressive suppression of photosynthesis caused by stomatal and non-stomatal limitations (Wise et al., 1992; Yordanov et al., 2003). Tolerant genotypes should not only be able to retain sufficient water under drought, but also have a highly active system for protection against oxidative stress injury.

Flooding affects about 10% of the global land area (Setter and Waters, 2003). In the USA alone 16% of soils are affected by waterlogging and the economic losses for crop production are estimated to be the second largest after drought (Zhou, 2010). Yield losses resulting from flooding depend on the plant species and age, soil type, and duration of flooding. Despite knowledge of adaptive mechanisms and regulation at the molecular level, understanding of the mechanisms behind plant response to flooding is very limited. Studies with Arabidopsis (*Arabidopsis thaliana*) (Gonzali et al., 2005) and rice (*Oryza sativa*) (Hattori et al., 2009; Xu et al., 2006; Singh et al., 2010) have shown that there are many genes associated with flooding responses suggesting that the regulation of flooding tolerance in plants is complex. Many studies have looked into the mechanisms underlying the responses to flooding stress using model plants (Vashisht et al., 2011) as well as crop species (Setter and Water, 2003; Zaidi et al., 2004; Rhine et al., 2010) however very few studies have looked at this at the whole plant and cellular level.

Plastoglobules are lipoprotein bodies attached to the thylakoids (Austin et al., 2006) that store lipids and antioxidants such as tocopherols, carotenes, and plastoquinones (Steinmuller and Tevini, 1985) and also contain tocopherol cyclase, which is involved in α-tocopherol synthesis (Austin et al., 2006; Vidi et al., 2006). Plastoglobules contain fibrillins, which are ubiquitous proteins that maintain plastoglobule structural integrity (Langenkamper et al., 2001; Vidi et al., 2006; Brehelin et al., 2007) and stabilize the photosynthetic apparatus during photo-oxidative stress (Yang et al., 2006; Youssef et al., 2010), osmotic stress (Gillet et al., 1998), drought (Rey et al., 2000), and low temperature (Rorat et al., 2001). Even though some studies have been conducted to dissect the role of plastoglobules in model (Ytterberg et al., 2006; Giacomelli et al., 2006) and some horticultural plants (Chen et al., 1998; Gillet et al., 1998), no information on major crops such as soybean is available for drought and flooding conditions.

Soybean is the world's most widely grown seed legume, providing an inexpensive source of protein and vegetable oil for human consumption. This important legume crop is adapted to grow in a wide range of climatic conditions; however, soybean growth, development, and yield are greatly affected by several abiotic stressors, such as; flooding (Komatsu et al., 2012; Khatoon et al., 2012), drought (Mohammadi et al., 2012), and salinity (Sobhanian et al., 2010). As in other major crops, breeding for drought tolerance in soybean has been a challenge because of the inherent complexity of breeding for drought tolerance combined with a lack of physiological perspective in the dissection of traits (Sadok and Sinclair, 2011) and limited drought tolerant germplasm resources (Carter et al., 1999, 2004). Traits that have been targeted for drought tolerance in soybean include deeper rooting system, sustained nitrogen fixation (Sinclair et al., 2007), slow canopy wilting (Sloane et al., 1990; Hufstetler et al., 2007; King et al., 2009) and water use efficiency. The slow wilting trait in soybean suggests a conservative water use strategy by some genotypes and has been used in breeding for drought tolerance. Even though there has been some success in breeding for abiotic stress tolerance in soybean, the underlying molecular and physiological mechanisms involved in drought and flooding tolerance are still poorly understood. Previous studies explored some morphological (Benjamin and Nielsen, 2006; Wang et al., 2012), physiological and biochemical (Sloane et al., 1990; Agarwal et al., 2005; Manavalan et al., 2009) and molecular (Ahuja et al., 2010; Stolf-Moreira et al., 2010; Manavalan et al., 2009) aspects in an effort to understand drought tolerance mechanisms in soybean; however, there is still limited information at the cellular and biochemical levels. Photosynthetic efficiency which is crucial for maximum yields is negatively affected by abiotic stress. In this study we looked at the role of some of the processes that will affect photosynthesis under drought and flooding stress. Using contrasting genotypes we compared soybean responses to drought and flooding stress at the physiological, biochemical, and cellular level, quantified these responses and tried to infer their role in soybean drought and flooding tolerance.

2. Materials and methods

2.1. Growing conditions

Four contrasting soybean (*Glycine max*) genotypes were used in this experiment: PI 567690 – drought tolerant (DT), Pana (PI 597387) – drought susceptible (DS) (Pathan et al., 2014), PI 408105A – flooding tolerant (FT) and S99-2281 (PI 654356) – flooding susceptible (FS). The plants were grown under a 14 h photoperiod and optimum temperature 28/18 °C day/night at the Division of Plant Sciences greenhouses, University of Missouri, Columbia. A mixture of soil and sand (2:1) was used in 26.5-L pots (top and bottom diameter were 30 cm and 27 cm, respectively, and 37 cm in height). Four seeds were sown per pot and Osmocote (slow release fertilizer – 14:14:14 – N:P_2O_5:K_2O; Scotts Co., Marysville, OH, USA) was used as a nutrient source at a rate of 20 g per pot. Pots were kept well-watered and thinning done to one plant per pot when the plants had two sets of unfolded trifoliate leaves (V2 stage). At the V5 stage (five unfolded trifoliate leaves), drought stress was imposed by withdrawing water and flooding stress was imposed by placing the pot with the plant into a 56.8-L pot (top and bottom diameter were 38 cm and 34 cm, respectively, and 46 cm in height) containing trash can liner. The 56.8-L pots were then filled with water to flood the 26.5-L pot. After 21 days of drought stress the plants were re-watered and allowed to recover. Flooding was done for 15 days and on day 16, the trash can liners were punctured to drain all water and the plants were allowed to recover. This experiment was set up as a randomized complete design with four replications.

2.2. Relative water content, chlorophyll content and gas exchange

Leaf relative water content (RWC) was determined using the equation: (Weatherley, 1950; Barr and Weatherley, 1962)

$$RWC = \frac{(FW - DW)}{(TW - DW)} \times 100$$

Where FW – leaf fresh weight, DW – leaf dry weight and TW – leaf turgid weight. Full leaves were used in the determination of RWC. To quantify variation in physiological traits (chlorophyll content and gas exchange) data were collected from an attached leaflet of the fourth trifoliate leaf from the main-stem apex at midday (11:00–13:30 h) at mild and severe stress. We used a self-calibrating chlorophyll meter (SPAD 502, Spectrum Technologies,

Plainfield, IL) to measure chlorophyll content based on SPAD values. A LI-COR 6400XT portable photosynthesis system (LI-COR, Lincoln, NE) was used to measure net photosynthetic rate, stomatal conductance, transpiration rate, and intercellular CO_2 (Ci). These measurements were taken at midday at the greenhouse temperature (28 °C) and ambient CO_2 conditions (300 μmol). The internal LED light source in the LI-COR 6400XT was set at 1600 μmol m^{-2} s^{-1} to have a constant and uniform light across all measurements.

2.3. Electron microscopy

At 21 days of drought and 16 days of flooding six leaf sections (1 mm^3) were collected at midday from an attached leaflet of the forth trifoliate leaf from the main apex and immediately fixed in 2% paraformaldehyde, 2% glutaraldehyde, in 100 μM sodium cacodylate buffer, pH 7.35 for sectioning. Fixed tissues were then rinsed with 100 μM sodium cacodylate buffer (pH 7.35) containing 10 μM 2-mercaptoethanol and 130 μM sucrose. Using a Pelco Biowave (Ted Pella, Inc. Redding, California), Secondary fixation was performed using 1% osmium tetroxide in 100 μM sodium cacodylate (pH 7.35) followed by rinsing with distilled water. A graded dehydration series was done in the Pelco Biowave using acetone and then dehydrated tissues were infiltrated with Epon/Spurr's resin and polymerized at 60 °C overnight. Sections were cut to a thickness of 85 nm using an ultramicrotome (Ultracut UCT, Leica Microsystems, Germany) with a diamond knife (Diatome, Hatfield, PA). These sections were stained using Sato's triple lead solution stain and 5% aqueous uranyl acetate (Sato, 1968). Images were acquired at 80 kV on the JEOL JEM 1400 transmission electron microscope (JEOL, Peabody, MA) equipped with an Ultrascan 1000 CCD (Gatan, Inc. Pleasanton, CA). Three sections were used per plant and a total of 30 chloroplasts per sample were used to determine the number of plastoglobules and starch granules while chloroplast size was determined from a total of 120 per genotype. ImageJ (ImageJ 1.46r, National Institutes of Health, USA) was used to determine chloroplast size.

2.4. Biochemical analysis

Leaf tissues were collected at midday from these genotypes after 20 days of drought stress and 15 days of flooding. The samples were immediately frozen in liquid nitrogen and stored at −81 °C until the time of processing.

2.4.1. Hormone analysis

For acidic hormones [Salicylic acid (SA), Abscisic acid (ABA), Jasmonic acid (JA), Jasmonoyl-isoleucine (JA-Ile), and 12-oxo-phytodienoic acid (OPDA)] assay, a liquid chromatography-mass spectrometry (LC-MS/MS) method was used. A mixture of deuterium labeled standards (D5SA, D6ABA, D2JA, and D5IAA) at 2.5 μM each was spiked at the beginning of the hormone extraction in each tube containing the frozen tissue sample. 900 μL of ice cold MeOH/ACN (1:1 v/v) was added to each sample. Samples were then homogenized with TissueLyserII for 2 min at a frequency of 15 Hz/sec and centrifuged at 16,000 g for 5 min at 4 °C. The supernatants were transferred to new 2 mL tubes and the pellets were re-extracted as previously described. The second supernatant was combined to the first one and dried down. The pellets were dissolved in 200 μL of 30% methanol and centrifuged again to remove un-dissolved material and the supernatant was transferred to vials for LC-MS/MS analysis.

The LC–MS/MS system used for hormone analysis is composed of a Shimadzu LC system with two Shimadzu solvent delivery pumps (model LC10AD), a Shimadzu integrated controller (SCL10Avp), a Valco two-position diverter valve, and a LEAP CTC PAL autosampler with a 50-ll sample loop. This LC system is interfaced with an AB Sciex 4000 QTRAP mass spectrometer equipped with a TurboIonSpray (TIS) electrospray ion source. Source parameters were set as follows: curtain gas, 25 arbitrary units (a.u.); source gas 1, 50 a.u.; source gas 2, 50 a.u.; collision activated dissociation, high; interface heater, on; temperature, 550 °C; and internal standard, −4.500. Both quadruples (Q1 and Q3) were set to unit resolution. Analyst software (version 1.4.2) was used to control sample acquisition and data analysis. The 4000 QTRAP mass spectrometer was tuned and calibrated according to the manufacturer's recommendations. Each hormone was detected using multiple reaction monitoring (MRM) transitions that were previously optimized using each standard and deuterium-labeled standard.

For LC separation, a monolithic C18 column (Onyx, 4.6 μm × 100 mm, Phenomenex, Torrance, California, USA) with a guard cartridge was used flowing at 1 mL min^{-1}. The gradient was from 60% solvent A (0.1% [v/v] acetic acid in Milli-Q water), held for 2 min, to 100% solvent B (90% acetonitrile [v/v] with 0.1% acetic acid [v/v] in 5 min). The LC was then ramped back to initial conditions (60% solvent A) for 1 min and re-equilibrated for an additional 2 min. For quantification, a series of standard samples containing different concentrations of hormones was prepared. The peak area in samples was first normalized in the same way as used for the standard samples and then quantified according to the standard curve.

2.4.2. Sugar analysis

The sugar standards, D-fructose, D-(+) glucose, sucrose, D-(+) raffinose pentahydrate, and stachyose hydrate, were purchased from Sigma Aldrich (St Louis, Missouri, USA). Acetonitrile, acetone, and deionized water were purchased from Fisher Scientific (Hampton, New Hampshire, USA). Compressed nitrogen of ultra-high-purity (UHP) grade was purchased from Praxair (Danbury, Connecticut, USA). The HPLC-ELSD system was Agilent 1200 series. Sugar standards of fructose, glucose, sucrose, raffinose, and stachyose were dissolved with water at a concentration of 10 mg/mL. A mixture of 1 mg/mL of the five sugar solution was then prepared and further diluted to 50, 100, 200, 300, 400, and 500 μg/mL in water.

Leaf samples weighing 0.5 g of were lyophilized for two days in a Labconco Freeze Dry System (FreeZone 6 L console freezer dry system with stoppering tray dryer, Labconco Corporation, Kansas City, Missouri, USA) and then ground using a Geno/Grinder2010 grinder (SPEX SamplePrep, Metuchen, New Jersey, USA). Sugar extraction and analysis were performed by a modified HPLC-ELSD method developed for soybeans at the Nguyen laboratory, University of Missouri (Valliyodan et al. unpublished).

2.5. RNA expression analysis

2.5.1. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis

100 mg of leaf tissue was used for RNA extraction using RNeasy Plant mini kit (Qiagen, CA, USA) according to the manufacturer's protocol. On-Column DNA digestion was performed by using RNase-Free DNase Set (Qiagen, CA, USA) according to the manufacturer's protocol. Total RNA (2 μg) from each sample was reverse-transcribed to cDNA in a 20 μL reaction volume using RNA to cDNA EcopryTM Premix (Double primed) cDNA Synthesis Kit (Clontech, CA, USA) according to the manufacturer's protocol. Quantitative RT-PCR (qRT-PCR) was performed using the cDNA product corresponding to 25 ng of total RNA in a 10 μL reaction volume using the Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo, USA) on

a detection system (ABI 7900HT (Life Technologies, Grand Island, New York, USA)). Three biological replicates and two technical replicates were used for the qRT-PCR analysis. The PCR conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, then 40 cycles of 95 °C for 15 s, 60 °C for 1 min. To normalize the gene expression, Actin (*Glyma 18g52780*) was used as an internal control. All novel primers were designed using Primer3 web-interface (Rozen and Skaletsky, 2000).

2.6. Data analysis

Data analysis was performed using PROC GLM for analysis of variance while PROC CORR and determining correlations (SAS version 9.3 – SAS Institute Inc., Cary, NC, USA). Least significant difference (LSD) at $\alpha = 0.05$ was used to determine significant differences among genotypes, treatments, and the interaction between the two. Statistical significance was based on a *P*-value of 0.05.

3. Results

Relative water content varied significantly among genotypes, with treatment as well as the interaction between genotype and treatment at $P = 0.0005$, <0.0001 and 0.0051 respectively (Table 1). At severe drought stress leaf relative water content ranged from 50.6 to 58.1% (Fig. 1A). Net photosynthesis, stomatal conductance, and internal carbon dioxide concentration (Ci) varied significantly among the genotypes studied ($P < 0.0001$, 0.0001 and 0.0165, respectively) and in response to stress ($P < 0.0001$) (Table 1). Variation in the number of plastoglobules and starch granules per μm^2 of chloroplast was highly significant ($P < 0.0001$) and variation in response to stress was highly significant at $P < 0.0001$. Drought and flooding tolerant genotype (PI 567690 and PI 408105A) seemed to have higher plastoglobule numbers (0.25 and 0.23 respectively) while the susceptible genotypes Pana (drought susceptible) and S99-2281 (flooding susceptible) had the low values (0.13 and 0.18 respectively).

Total sugars varied significantly among the genotypes ($P = 0.0271$) and with treatment ($P < 0.0001$) (Table 1) while for hormones analyzed there was significant variation among the genotypes for ABA, OPDA and SA at $P = 0.0417$, 0.0014 and <0.0001 respectively. Treatment effects were highly significant ($P < 0.0001$) for all the hormones except JA-ILE (Table 1).

3.1. Physiological responses

Drought stress reduced chlorophyll content by 6–13% while flooding stress resulted in a reduction of 18–34% (Fig. 1B). PI 408105A (FT) recorded the lowest reduction under drought and flooding stress (6 and 18% respectively) while Pana (DS) had the highest (13 and 34% respectively). Stomatal conductance and net photosynthesis were negatively affected by drought and flooding with drought having the highest effects for most of the genotypes (Fig. 2A, B and Table 2). PI 567690 (DT) had a 30%, 28% reduction in net photosynthesis and 28%, 10% reduction in stomatal conductance while Pana had a 52%, 37% reduction in net photosynthesis and 63%, 35% reduction in stomatal conductance under drought and flooding stress respectively. PI 408105A suffered a 42%, 29% reduction in net photosynthesis and a 42%, 17% reduction in stomatal conductance under drought and flooding stress respectively when compared to a reduction of 65%, 39% in net photosynthesis and 81%, 44% reduction in stomatal conductance for S99-2281 (FS).

3.2. Cellular responses

3.2.1. Plastoglobule and starch granules

Plastoglobules and starch granules were affected by drought and flooding stress with drought increasing the number of plastoglobules while flooding increase the number of starch granules (Fig. 3). Drought stress increased the number of plastoglobules by 34% in PI 567690 (DT), 36% in PI 408105A (FT) and 28% in S99-2281 (FS) but resulted in a 62% decrease in Pana (DS) (Fig. 4A, Table 2).

Table 1
Mean values based on analysis of variance showing variation in physiological and anatomical traits among genotypes. Means with the same letter indicates no difference between them based on $LSD_{(\alpha = 0.05)}$ value.

1. Physiological Traits		Relative water content	Net photosynthesis ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)	Stomatal conductance ($mmol\ m^{-2}\ s^{-1}$)	Intercellular CO_2 ($\mu mol\ CO_2\ mol\ air^{-1}$)	Plastoglobules (μm^{-2})	Starch granules (μm^{-2})
Genotype	Characteristic	(%)					
PI 567690	Drought tolerant[a] (DT)	70.28^{A}	11.82^{A}	0.244^{A}	190.4^{A}	0.25^{A}	0.08^{B}
PANA	Drought susceptible (DS)	66.36^{B}	8.91^{B}	0.155^{B}	165.5^{B}	0.13^{C}	0.11^{A}
PI 408105A	Flooding tolerant (FT)	69.98^{A}	10.46^{A}	0.210^{A}	175.7^{A}	0.23^{A}	0.07^{B}
S99-2281	Flooding susceptible (FS)	66.74^{B}	8.32^{B}	0.128^{B}	162.8^{B}	0.18^{B}	0.04^{C}
$LSD_{(\alpha = 0.05)}$		3.06	2.56	0.056	17.56	0.033	0.017
Genotype		**	***	**	*	***	<0.0001
Treatment		***	***	***	***	***	<0.0001
Genotype*treatment		*	NS	NS	NS	***	<0.0001

2. Biochemical variation		Fructose	Glucose	Sucrose	Raffinose	Stachyose	Total sugars	ABA	JA	JA-ILE	OPDA	SA
Genotype	Characteristic	($mg\ g^{-1}$ of dry weight leaf tissue)						($ng\ g^{-1}$ dry weight leaf tissue)				
PI 567690	Drought tolerant (DT)	23.31^{B}	9.61^{A}	10.28^{A}	4.66^{A}	4.48^{A}	52.32^{B}	5621.7^{A}	33.44^{A}	14.28^{A}	645.3^{B}	21348^{B}
PANA	Drought susceptible (DS)	22.30^{B}	8.49^{BC}	$8.42B^{A}$	4.53^{BA}	4.40^{A}	47.98^{B}	3460.0^{B}	31.01^{A}	11.38^{A}	731.3^{B}	9601^{C}
PI 408105A	Flooding tolerant (FT)	36.58^{A}	10.10^{A}	6.83^{B}	4.45^{BA}	4.27^{A}	62.22^{A}	5584.2^{A}	39.85^{A}	38.42^{A}	978.7^{A}	18865^{B}
S99-2281	Flooding susceptible (FS)	23.05^{B}	7.82^{C}	10.01^{A}	4.34^{B}	4.40^{A}	49.61^{B}	5482.9^{A}	44.62^{A}	17.93^{A}	1021.3^{A}	27126^{A}
$LSD_{(\alpha=0.05)}$		4.46	1.26	2.42	0.31	0.30	5.80	1866.3	15.70	29.71	120.16	4044.1
Genotype		***	*	*	NS	NS	**	*	NS	NS	**	***
Treatment		***	NS	*	*	*	***	***	***	NS	***	*
Stress level		***	***	***	***	***	***	NS	NS	NS	***	*
Genotype*treatment		*	*	NS	NS	NS	NS	NS	*	NS	**	**
Genotype*stress level		NS	NS	*	NS	NS	NS	NS	NS	NS	NS	NS

*, **, *** denotes significance at $P < 0.05$, 0.001, 0.0001 respectively, NS denotes no significant difference.
[a] A genotype exhibiting the slow canopy wilting phenotype (Pathan et al., 2014).

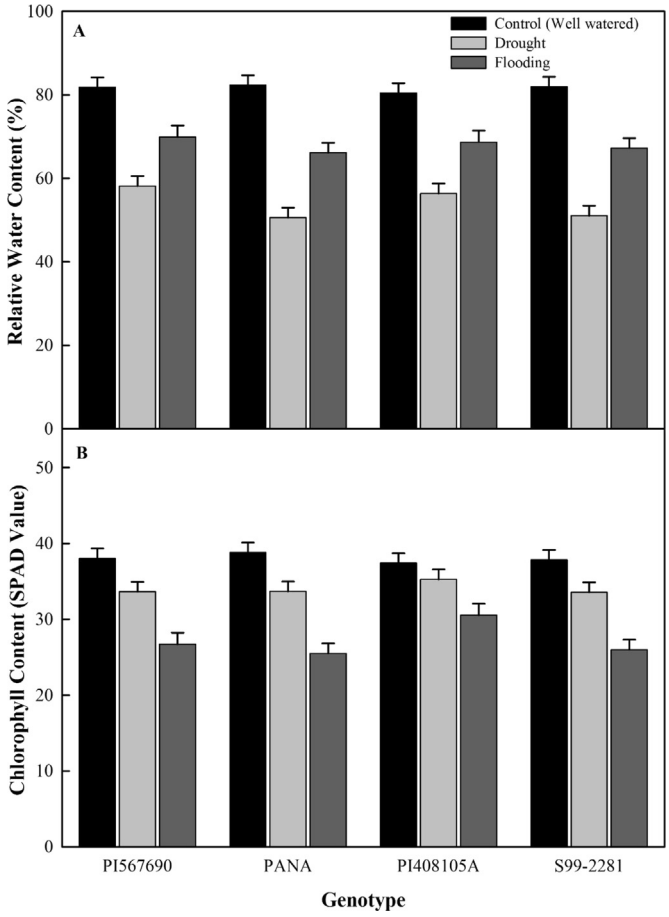

Fig. 1. Relative water content (%) and chlorophyll content among genotypes under drought and flooding stress (PI 567690 – DT, Pana – DS, PI 408105A – FT, S99-2281 – FS).

Plastoglobule numbers were not affected by flooding in all genotypes except Pana which recorded a 71% decrease. Under drought conditions starch granules were significantly reduced in PI 567690 (97%) and S99-2281 (96%) while only PI 408105A recorded a decline (98%) under flooding stress with significant increases in PI 567690 (39%) and S99-2281 (76%) (Fig. 4B, Table 2).

3.3. Fibrillin proteins

The relative expression level for all the seven fibrillin proteins was significantly higher in the tolerant genotypes compared to the susceptible genotypes (Fig. 5). Three of these fibrillin proteins were highly expressed under both drought and flooding stress with higher expression levels under drought stress compared to flooding stress (x25.17 and x3.90 being the highest values for drought and flooding stress, respectively). In PI 567690 (DT), fibrillin1a was highly expressed under drought stress while the level of expression for fibrillin1b and fibrillin7a were similar but down-regulated in Pana (DS). Under flooding conditions fibrillin1b and fibrillin7a were highly up-regulated in PI 408105A (FT) (x4 and x3, respectively) when compared to x1 for fibrillin1b and 7a while fibrillin1a was down-regulated (x0.8). Fibrillins 2, 3a, 4, and 8 were expressed at similar levels under both drought and flooding stress in PI 567690 and PI 408105A (x1–x3) but were all down-regulated in Pana (x0.004–x0.03). In genotype S99-2281 (FS), fibrillin2 and 8 were slightly up-regulated (x1.4 and x1.5 respectively) while fibrillin3a was slightly down-regulated (Fig. 5).

3.4. Biochemical responses

3.4.1. Soluble sugars

Sugar concentration in the leaves was significantly affected by drought and flooding stress among the genotypes (Fig. 6). Under drought stress, PI 567690 (DT) and PI 408105A (FT) showed a significant increase in total sugar concentration (Fig. 6A). Fructose formed a large percentage (36–65%) of the soluble sugars and was also significantly increased in these two genotypes (Fig. 6B). Sucrose concentration was significantly increased in the genotype PI 567690 under drought stress (Fig. 6D) while stachyose was significantly decreased (Fig. 6F). Flooding stress significantly increased glucose concentration but decreased stachyose concentration in PI 567690 (Fig. 6C and F) and sucrose concentration in S99-2281 (FS) (Fig. 6D). Both drought and flooding stress slightly decreased raffinose concentration in all the genotypes (Fig. 6E).

3.4.2. Hormones

Drought and flooding stress had significant effects on leaf hormone concentrations among all the genotypes (Fig. 7). There was a significant increase in total leaf hormones concentration in genotypes PI 408105A (FT) and S99-2281 (FS) in response to flooding stress and a decrease in genotypes PI 567690 (DT) and PI 408105A due to drought stress (Fig. 7A). Under drought stress Abscisic acid (ABA) was significantly increased in all genotypes (Fig. 7B) while Jasmonic acid (JA) and 12-oxo-phytodienoic acid (OPDA) decreased in all the genotypes (Fig. 7C, E). Jasmonoyl-isoleucine (JA-ILE) and Salicylic acid (SA) concentration was reduced in all genotypes except Pana (DS) and S99-2281, respectively (Fig. 7D, F).

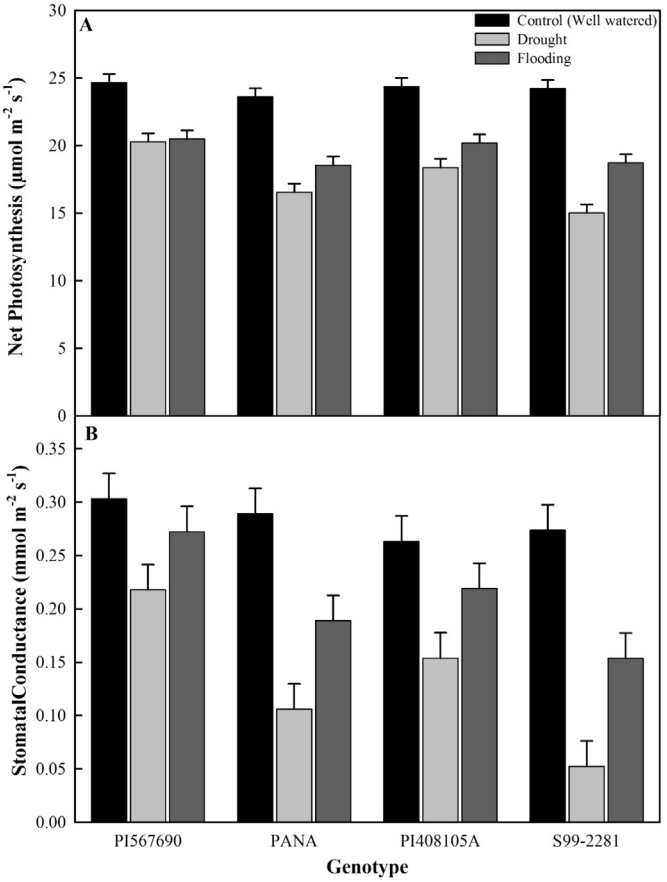

Fig. 2. Effects of drought and flooding stress on net photosynthetic rate and stomatal conductance of contrasting soybean genotypes (PI 567690 – DT, Pana – DS, PI 408105A – FT, S99-2281 – FS).

Table 2
Physiological, biochemical and fibrillin proteins variations among contrasting soybean genotypes in response to drought and flooding stress (DT – drought tolerant, DS – drought susceptible, FT – flooding tolerant, FS – flooding susceptible).

	Drought stress				Flooding stress			
	PI 567690 (DT)	Pana (DS)	PI 408105A (FT)	S99-2281 (FS)	PI 567690 (DT)	Pana (DS)	PI 408105A (FT)	S99-2281 (FS)
Chlorophyll content	Decrease (12%)	Decrease (13%)	Decrease (6%)	Decrease (11%)	Decrease (30%)	Decrease (34%)	Decrease (18%)	Decrease (31%)
Net Photosynthesis	Decrease (30%)	Decrease (52%)	Decrease (42%)	Decrease (65%)	Decrease (28%)	Decrease (37%)	Decrease (29%)	Decrease (39%)
Stomatal conductance	Decrease (28%)	Decrease (63%)	Decrease (42%)	Decrease (81%)	Decrease (10%)	Decrease (35%)	Decrease (17%)	Decrease (44%)
Plastoglobules	Increase (34%)	Decrease (62%)	Increase (36%)	Increase (28%)	NS	Decrease (71%)	NS	NS
Starch Granules	Decrease (97%)	NS	NS	Decrease (96%)	Increase (39%)	NS	Decrease (98%)	Increase (76%)
Fibrillin proteins								
• Fibrillin1a (FBN1a)	Expressed (x25)	Suppressed (x0.03)	–	–	–	–	Expressed (x2.9)	Suppressed (x0.8)
• Fibrillin1b (FBN1b)	Expressed (x11)	Suppressed (x0.01)	–	–	–	–	Expressed (x3.9)	Expressed (x1.1)
• Fibrillin2 (FBN2)	Expressed (x2)	Suppressed (x0.01)	–	–	–	–	Expressed (x2.4)	Expressed (x1.4)
• Fibrillin3a (FBN3a)	Expressed (x4)	Suppressed (x0.01)	–	–	–	–	Expressed (x2.5)	Expressed (x1.0)
• Fibrillin4 (FBN4)	Expressed (x3)	Suppressed (x0.01)	–	–	–	–	Expressed (x2.2)	Suppressed (x0.8)
• Fibrillin7a (FBN7a)	Expressed (x13)	Suppressed (x0.01	–	–	–	–	Expressed (x3.8)	Expressed (x1.3)
• Fibrillin8 (FBN8)	Expressed (x1.4)	Suppressed (x0.004)	–	–	–	–	Expressed (x1.6)	Expressed (x1.2)
Sugars								
• Fructose	Increase (132%)	Increase (39%)	Increase (87%)	Increase (44%)	NS	NS	NS	NS
• Glucose	Increase (22%)	NS	NS	NS	Increase	NS	NS	NS
• Sucrose	Increase	Increase	Increase	Increase	NS	NS	Increase	Decrease
• Raffinose	Decrease	Decrease	Decrease	Decrease	Decrease	Decrease	Decrease	Decrease
• Stachyose	Decrease	Decrease	Decrease	Decrease	Decrease	Decrease	Decrease	Decrease
Hormones								
• Abscisic acid (ABA)	Increase (97%)	Increase (111%)	Increase (24%)	Increase (98%)	Decrease (27%)	NS	Decrease (66%)	Decrease (18%)
• Jasmonic Acid (JA)	Decrease (61%)	Decrease (10%)	Decrease (60%)	Decrease (57%)	Decrease (42%)	Increase (22%)	Increase (136%)	NS
• Jasmonoyl isoleucine (JA-ILE)	Decrease (70%)	Increase (26%)	Decrease (34%)	Decrease (21%)	Decrease (70%)	Increase (67%)	Increase (930%)	NS
• 12-oxo-phytodienoic acid (OPDA)	Decrease (37%)	Decrease (34%)	Decrease (52%)	Decrease (28%)	NS	Increase (40%)	Increase (67%)	Increase (133%)
• Salicylic acid (SA)	Decrease (38%)	Decrease (18%)	Decrease (44%)	Increase (43%)	Decrease (23%)	NS	Increase (45%)	Increase (55%)

Total hormone concentration was significantly increased in PI 408105A and S99-2281 but decreased in PI 567690 under flooding stress. All genotypes except Pana recorded significant decrease in ABA concentration under flooding conditions (Fig. 7B) while JA increased in all genotypes except PI 567690 (Fig. 7C). Flooding also resulted in a significant increase of JA-ILE concentration for Pana and PI 408105A and a decrease in PI 567690 (Fig. 7D). Flooding stress also increased OPDA concentration in all genotypes except PI 567690 (Fig. 7E) while SA was decreased in PI 567690 but increased in PI 408105A and S99-2281 (Fig. 7F).

4. Discussion

Changes in water status (drought or flooding) have an impact on the plant growth and development. Although these abiotic stresses result in a decrease in photosynthetic rate, our study revealed that different mechanisms are responsible for this reduction.

4.1. Mechanisms involved in drought stress responses

Under drought stress, higher leaf ABA levels in the leaves decreased stomatal conductance among all genotypes and reduced net photosynthesis except in genotype PI 408105A (FT) (Figs. 2 and 7 and Table 2). By facilitating gas diffusion, open stomata allow CO_2 to reach sites of photosynthesis, while at the same time allowing water vapor to exit the leaf to the atmosphere. A decrease in soil water potential or an increase in atmospheric vapor pressure deficit (VPD) induces a hydraulic cascade of water potential drops in a plant causing reduced stomatal conductance. There is also a biochemical response in stomatal closure when plants are exposed to drought stress causing stomatal closure even in absence of any change in leaf water potential (Gollan et al., 1986). ABA is synthesized in roots (Simonneau et al., 1998) and shoots (Christmann et al., 2005, 2007) and transported to the guard cells where it induces stomatal closure (Davies and Zhang, 1991). A reduction in stomata opening will result in a decline in the amount of CO_2 assimilated into the leaf and this may cause a decline in net photosynthesis. Our results have shown that an increase in leaf ABA concentration coincided with a decrease in stomatal conductance and this reduced net photosynthesis. Our results also show that the drought tolerant genotype PI 567690 had a higher leaf ABA concentration than the drought susceptible genotype (Pana). This agrees with the findings in rice (*Oryza sativa*) (Perales et al., 2005) and barley (*Hordeum vulgare* L.) (Veselov et al., 2008; Thameur et al., 2011) showing that drought tolerant genotypes accumulate more ABA than the susceptible ones.

We observed an increased number of plastoglobules in response to drought stress in all genotypes except the drought susceptible Pana (Figs. 3 and 4A). Similar responses have been reported in Tobacco (*Nicotiana tabacum*) under drought (Rey et al., 2000) and salt (Locy et al., 1996) stress as well as tomato (*Lycopersicum esculentum* Mill. cv. Moneymaker) in the presence of heavy metals (Baszynski et al., 1980). This strongly suggests the involvement of the plastoglobules in plant responses to stress. There is also evidence linking plastoglobulins in jasmonate biosynthesis (Youssef et al., 2010) or plastoquinone accumulation (Singh et al., 2010, 2012), pointing to a possible role in stress tolerance. To date, the biochemical or physiological mechanism explaining the role of plastoglobulins in plant adaptation to abiotic stress remains poorly understood.

Yang et al. (2006) suggested that ABA induces fibrillin accumulation and the ABA response regulators (ABI1, ABI2) regulate fibrillin expression. Fibrillins associate with stromal lamellae of thylakoids, which transports nutrients and substances needed in the thylakoids in order to keep the organelle alive and functioning, and fibrillic carotenoid-containing structures of chromoplasts (Rey et al., 2000), and are known to accumulate during abiotic stress (Manach and Kuntz, 1999; Gillet et al., 1998). Although there is evidence of an increase in plastoglobule number and size in

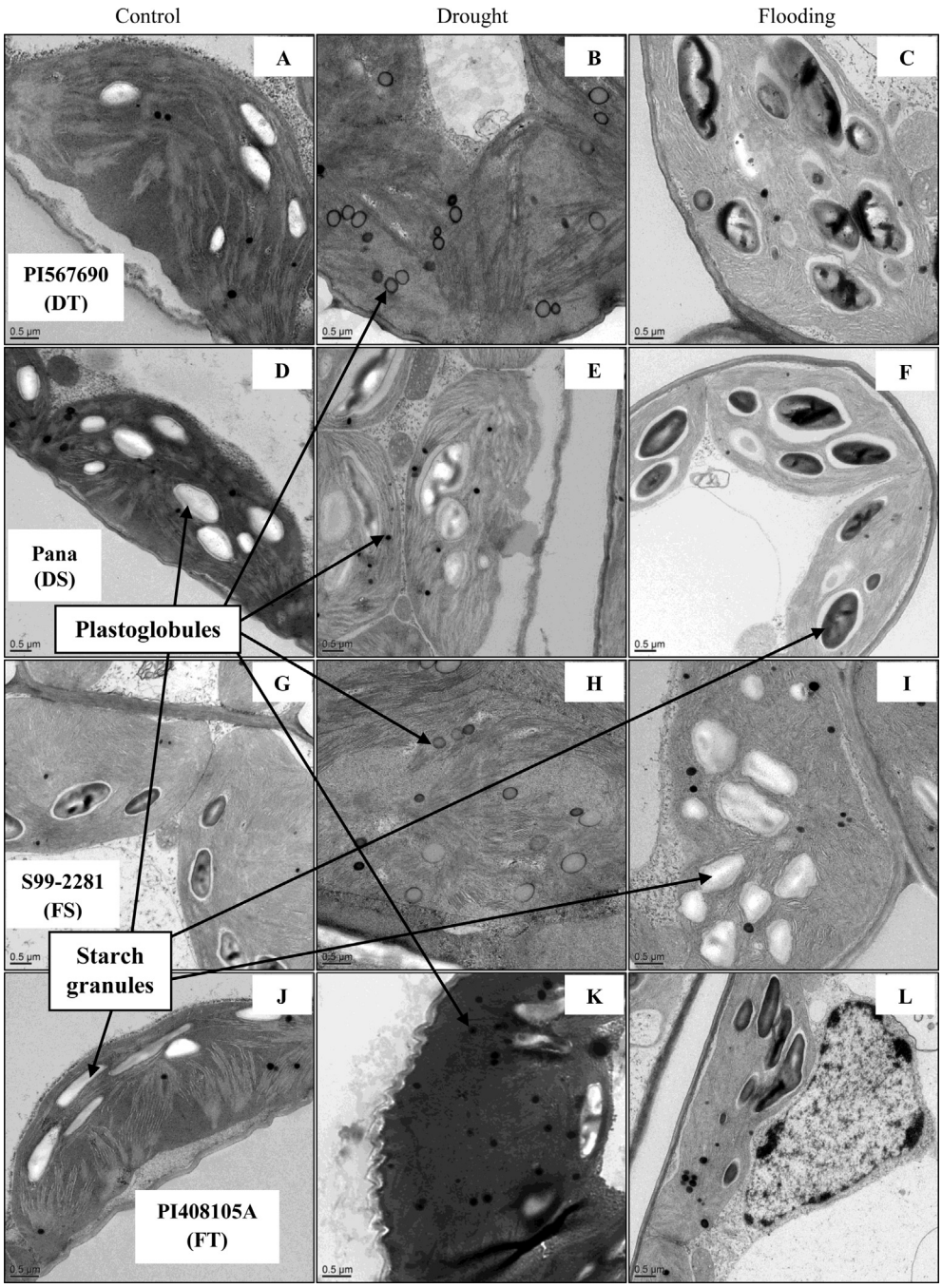

Fig. 3. Variation in starch granules and plastoglobule number among contrasting soybean genotypes in response to drought and flooding stress (PI 567690 – DT, Pana – DS, PI 408105A – FT, S99-2281 – FS).

response to abiotic stress the regulatory mechanisms are still elusive. This study shows that under drought stress, reduction in photosynthetic rate is mainly due to reduced stomatal conductance caused by increased leaf ABA concentration. ABA levels in the leaves are also important for drought tolerance in soybean.

4.2. Effects of drought and flooding stress on sugar metabolism

In our study, fructose accounted for much of the significant increase in leaf total sugars under drought stress while there was no significant variation under flooding stress (Fig. 6B). Some studies have pointed out that soluble sugar changes do not follow a static model and may vary with the genotype and the stress factor (Castonguay et al., 1995; Morsy et al., 2007). Gupta and Kaur (2005) suggested that sucrose and glucose either act as substrates for cellular respiration or osmolytes to maintain cell homeostasis while fructose is involved in the synthesis of secondary metabolites as well as erythrose-4-P, which acts as a substrate in lignin and phenolic compounds synthesis (Rosa et al., 2009). Even though all this suggests that under stress conditions the metabolism of soluble sugars is a dynamic process simultaneously involving degrading and synthetic reactions, our study shows that

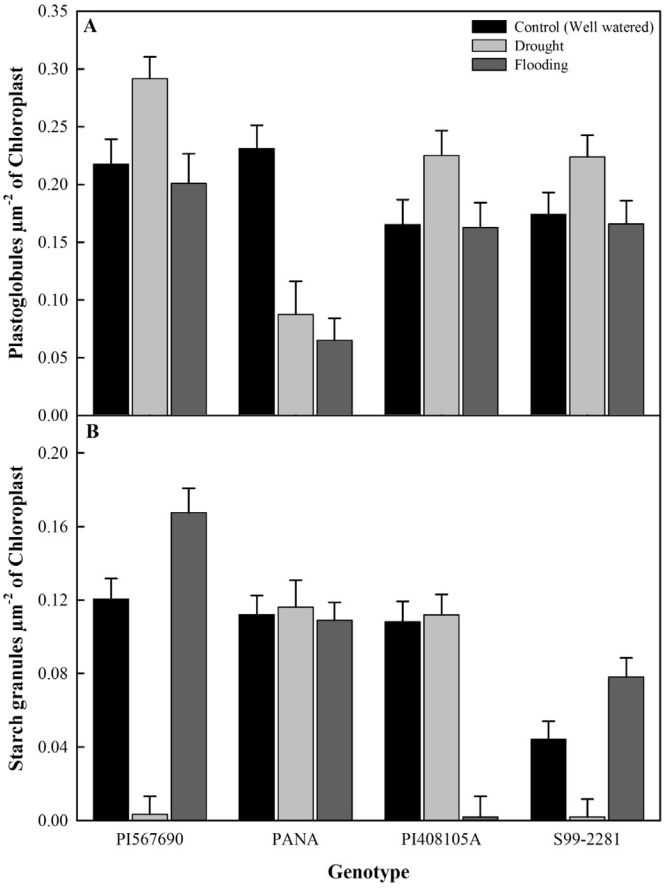

Fig. 4. Effects of drought and flooding stress on number of plastoglobules and starch granules in contrasting soybean leaves (PI 567690 – DT, Pana – DS, PI 408105A – FT, S99-2281 – FS).

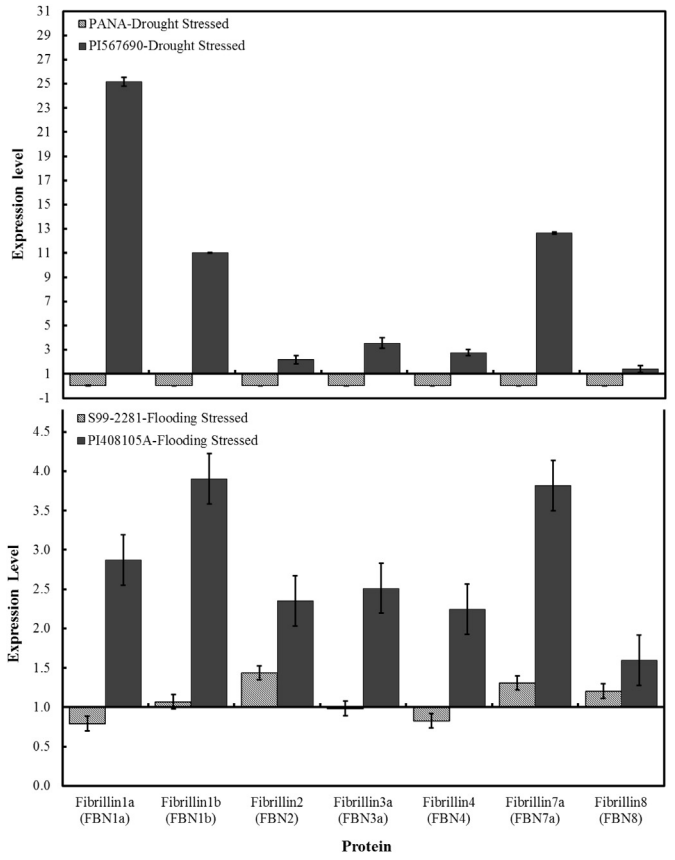

Fig. 5. Relative expression of fibrillin proteins in contrasting soybean genotypes under drought and flooding stress (PI 567690 – DT, Pana – DS, PI 408105A – FT, S99-2281 – FS). Fibrillin1a (FBN1a) – *Glyma15g11910.1*, Fibrillin1b (FBN1b) – *Glyma09g01080.1*, Fibrillin2 (FBN2) – *Glyma01g01910.1*, Fibrillin3a (FBN3a) – *Glyma15g05290.1*, Fibrillin4 (FBN4) – *Glyma20g34960.1*, Fibrillin7a (FBN7a) – *Glyma02g29260.1*, Fibrillin8 (FBN8) – *Glyma07g00410.1*.

drought resulted in an increase in total sugars accumulation in the leaves in soybean (Fig. 6). This finding agrees with the suggested role of soluble sugars as signaling molecules under stress (Chaves and Oliveira, 2004) and their interaction with hormones as part of the sugar sensing and signaling network in plants (Rolland et al., 2006).

4.3. Mechanisms involved in flooding stress responses

Even though we did not see significant reductions in stomatal conductance under flooding stress for all genotypes compared to drought stress, net photosynthesis was reduced in the susceptible genotypes (Pana and S99-2281) (Fig. 2). Under drought stress there was a clear relationship between leaf ABA concentration, stomatal conductance, and net photosynthesis but this does not seem to be the case for flooding stress (Figs. 3 and 7 and Table 2). This suggests that there are different mechanisms responsible for reduction in net photosynthesis under flooding stress. Oxygen deficiency due to a reduction in stomatal aperture can induce a reduction in the rate of photosynthesis (Huang et al., 1997; Malik et al., 2001). Other factors that contribute to this include reduction in leaf area, decline in chlorophyll content and early leaf senescence (Sena and Kozlowski, 1980), reduced sink demand (Wample and Thornton, 1984) and disruption in photosynthate transport (Sij and Swanson, 1973).

Our results show that flooding stress significantly ($P < 0.0001$) increased leaf starch granules in PI 567690 and S99-2281 but these were significantly reduced in PI 408105A (Tables 1 and 2 and Figs. 3 and 4B). Under flooding conditions, decreased rate of phloem transport to the roots results in starch accumulation (Topa and Cheeseman, 1992). Flooding stress may also lead to the inhibition of photosynthetic activity in the mesophyll as well as reductions in the metabolic activity and translocation of photoassimilates (Drew, 1997; Sachs and Vartapetian, 2007). In our study the accumulation of starch in the leaves played an important role in reduction of photosynthesis under flooding stress.

4.4. Fibrillin genes and their expression during stress conditions

Based on the protein sequence of soybean fibrillin genes, the seven genes differed in their amino acid content, which ranged from 265 to 370 with low conserved sequences (Supplementary data 1) and their clustering is provided (Supplementary data 2). Under drought stress, all seven fibrillin genes were up-regulated in the drought tolerant genotype PI 567690 and down-regulated in Pana, which is a drought susceptible genotype (Fig. 5). Highly expressed fibrillin genes were FBN1a (*Glyma15g11910*), FBN1b (*Glyma09g01080*), and FBN7a (*Glyma02g29260*). Under flooding stress, expression levels for the seven fibrillin genes were lower

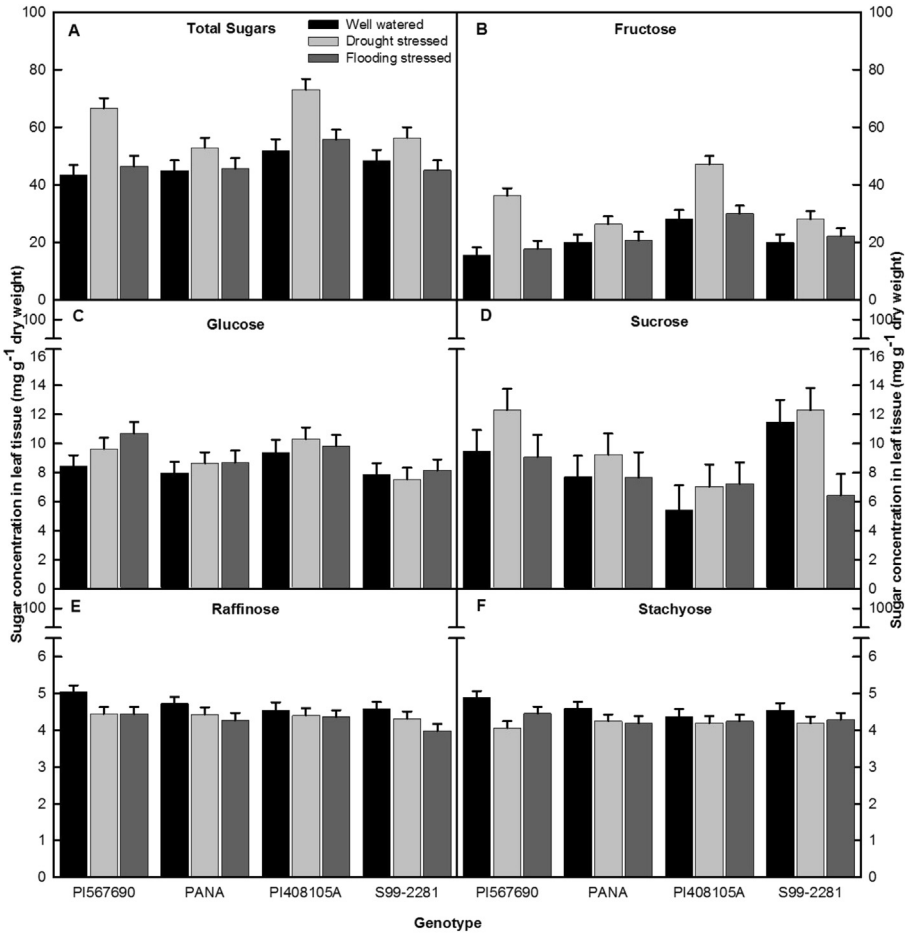

Fig. 6. Effects of drought and flooding stress on sugar concentration in the leaves of contrasting soybean genotypes (PI 567690 – DT, Pana – DS, PI 408105A – FT, S99-2281 – FS).

compared to drought. All seven genes were up-regulated in the flooding tolerant genotype (PI 408105A); whereas, only three of them [FBN2 (*Glyma01g01910*), FBN7a (*Glyma02g29260*) and FBN8 (*Glyma07g00410*)] were up-regulated in the susceptible genotype (S99-2281). This suggests that fibrillin proteins are involved in plant response to drought or flooding stress. A study by Giacomelli et al. (2006) using Arabidopsis demonstrated that FBN1a, FBN1b, FBN2, FBN4, and FBN7 were consistently 2- to 10-fold up-regulated in response to the transition to high light conditions. Yang et al. (2006) also found that FBN1a and FBN1b were correlated with protection against photoinhibition under high light. The presence of FBN4 in the PSII light-harvesting complex, plastoglobule, thylakoid membrane system, as well as the presence of a conserved lipocalin signature in FBN4 (Jones et al., 2006), indicates a possible role of this fibrillin protein in plastoglobule development and oxidative stress. In addition, FBN4 could be involved in the delivery of lipophilic antioxidants to particular locations, such as the PSII light-harvesting complex, within the photosynthetic membrane system (Singh et al., 2010).

Overexpression of FBN1a in Arabidopsis enhances photosystem II (PSII) photo-tolerance, while reduced FBN1a expression decreases PSII photo-tolerance (Yang et al., 2006). Simultaneous knockdown of the Arabidopsis FBN1-2 fibrillin family using RNA interference causes increased sensitivity to photo-inhibition under conditions of high light intensity combined with cold, which triggers oxidative stress (Youssef et al., 2010). Knockdown of FBN4 expression in apple and mutation of FBN4 in Arabidopsis resulted in increased sensitivity to ozone, intense light and paraquat, and resulted in higher levels of superoxide anion production during paraquat treatment (Singh et al., 2010). Our study suggests the possible involvement of these seven fibrillin genes, Fibrillin1a, 1b, 2, 3a, 4, 7a and 8, in plant responses to drought and flooding stress. Although fibrillin accumulation is induced in response to abiotic stresses, its role in plant response to stress and the molecular mechanism regulating its accumulation are still elusive.

5. Conclusion

Drought and flooding stress reduces photosynthetic rate in soybean. Under drought stress, reduction in photosynthetic rate is mainly due to a reduction in stomatal conductance caused by increased ABA concentration in the leaves. Different studies have shown that there are several factors that may cause the reduction in photosynthetic rate under flooding conditions, however our results indicate that starch accumulation in leaves play a key role in this reduction. Under stress conditions, metabolism of soluble sugars is a dynamic process which may involve simultaneous degrading and synthetic reactions and therefore levels of soluble sugars may not be a suitable indicator of mechanisms contributing towards stress tolerance. Plastoglobules play a role in abiotic stress tolerance although the mechanisms regulating this are still unclear. This can be supported by the observed increase in plastoglobule number and size in response to stress and the observed differences in plastoglobule numbers among tolerant and

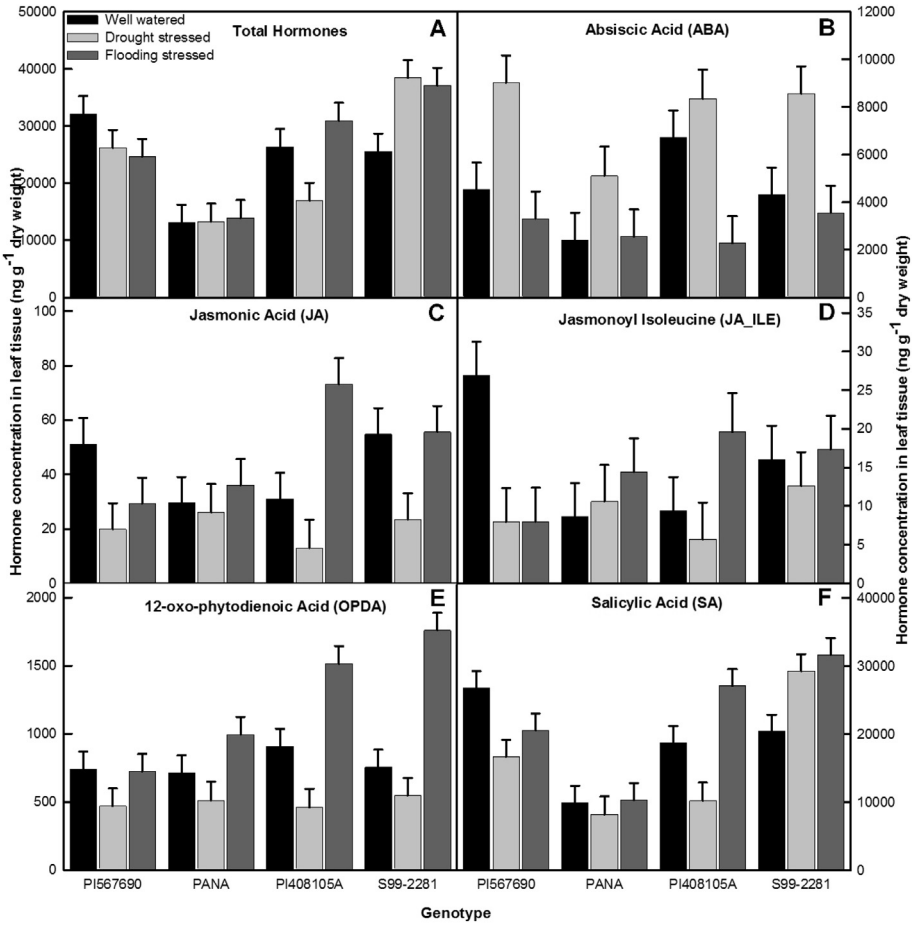

Fig. 7. Effects of drought and flooding stress on different hormones in the leaves of contrasting soybean genotypes (PI 567690 – DT, Pana – DS, PI 408105A – FT, S99-2281 – FS).

susceptible genotypes. There is also a possible involvement of fibrillin proteins/genes in plant response to drought and flooding stress. Our study highlights some key aspects that are involved in plant response to abiotic stress with findings that raise some key questions regarding the actual role and function of plastoglobules in abiotic stress tolerance.

Authors' contribution

1. Raymond N. Mutava, Silvas Jebakumar Prince K., Naeem Hasan Syed: Contributed equally in objectives formulation, experimental design and set up, data collection, analysis and interpretation.

2. Li Song and Wei Chen: Did all tissue collection, DNA extraction and qRT-PCR analysis.

3. Babu Valliyodan: Coordinated biochemical analysis.

4. Henry T. Nguyen: Provided guidance and all other support that was needed to complete this research.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2014.11.010.

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